48. A method for increasing the sensitivity of a tumor cell to a chemotherapeutic agent, comprising exposing said cell to the pharmaceutical composition of claim 43.

49. A method for increasing the sensitivity of a tumor cell to a chemotherapeutic agent, comprising exposing said cell to the pharmaceutical composition of claim 44.

50. A method for increasing the sensitivity of a tumor cell to a chemotherapeutic agent, comprising exposing said cell to the pharmaceutical composition of claim 45.

51. A method for increasing the sensitivity of a tumor cell to a chemotherapeutic agent, comprising exposing said cell to the pharmaceutical composition of claim 46.

REMARKS

Claims 1-12, 14, 15, 17 and 19-26 were pending in this application. Claims 1-12, 14, 15, 17 and 19-26 have been canceled without prejudice to Applicant's right to pursue the subject matter of any canceled claims in subsequent applications.

New claims 27-51 have been added. Support for the new claims can be found in the specification as originally filed as set forth in the chart below. The new claims do not constitute new matter under 35 U.S.C. § 132. All claims pending as of entry of the instant amendment are attached hereto as Exhibit A.

Claim	Support in Specification
27	page 1, lines 8-10; page 3, lines 20-22; page 11, lines 32-35; page 16, lines 11-15
28	page 4, lines 6-13
29, 32, 35, 37, 39	page 6, lines 3-13

30	page 1, lines 8-10; page 4, lines 16-19; page 11, lines 32-35; page 16, lines 11-15
31	page 4, lines 19-26
33	page 1, lines 8-10; page 4, lines 27-30; page 11, lines 32-35; page 16, lines 11-15
34	page 4, line 30 to page 5, line1
36	page 1, lines 8-10; page 11, lines 32-35; page 12, lines 17-21; page 16, lines 11-15
38	page 1, lines 8-10; page 11, lines 32-35; page 16, lines 11-15; page 20, lines 10-19
40	page 8, lines 7-12
41	page 10 line 30 to page 11, line 21
42-46	page 5, lines 11-15; page 14, line 30 to page 15, line 5
47-51	page 5, line 18 to page 6, line 2

The Rejections Under 35 U.S.C. § 112, First Paragraph Should Be Withdrawn

Claims 8-12, 14, 15, 17 and 19-26 stand rejected under 35 U.S.C. §112, first paragraph for containing subject matter not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Specifically, the Examiner states that the rejected claims "do not overcome the unpredictability in the field of antisense design that any nucleotide 10-40 bases would specifically bind and inhibit the target bcl-2 gene for the functions claimed." (February 8,

2002 Office Action at p. 3).

New claims 27-53 more particularly point out and distinctly claim that which Applicants regard as the claimed invention. Specifically, the claimed methods and pharmaceutical compositions relate to oligonucleotides that are defined as hybridizing to strategic sites of the bcl-2 sequence such as a translation initiation, splice donor or splice acceptor sequence of SEQ ID NO:19; or hybridizing to at least one of the first six codons of the reading frame of SEQ ID NO:19, or to the 5'-cap region of SEQ ID NO:19. The specification provides that "[b]locking translation at such strategic sites prevents formation of the functional bcl-2 gene product." (p. 12 of the instant specification). These "strategic sites" are clearly defined by the specification and by the claims. *See, e.g.*, the instant specification describes targeting the first six codons of bcl-2 in the treatment of lymphoma cell (p. 46, lines 4-6 of the instant specification); other preferred examples of regions of the bcl-2 gene to be targeted include the translation initiation site, splice donor and splice acceptor sequence of SEQ ID NO:19 (Table 1, p. 13 of the instant specification); and the translation initiation site or the 5'-cap region of SEQ ID NO:19 (p. 33 of the instant specification). The instant specification also provides evidence of the efficacy of targeting these regions. Examples 2-18, which demonstrate the efficacy of the claimed oligonucleotides in reducing the level of bcl-2 and in killing cancer cells, in particular lymphoma cells (pp. 21-57 of the specification).

Further, the efficacy of the claimed methods and pharmaceutical compositions for the treatment of cancer has been established by the post-filing art, thereby demonstrating that the instant invention was fully enabled at the time of filing. In particular, it has been shown that the pharmaceutical compounds and methods of the instant invention reduced tumor mass and led to an improvement in symptoms for cancer patients. (Webb et al., 1997, "BCL-2 antisense therapy in patients with non-Hodgkin lymphoma," Lancet 349:1137-41). Moreover, Delihas has described numerous clinical trials that are underway on various types of cancer using the

compositions and methods of the instant invention. (Delihas, 2001, "Targeting the expression of anti-apoptotic proteins by antisense oligonucleotides," Curr. Drug Targets 2:167-80). A number of studies have also shown that the claimed invention can significantly increase the sensitivity of cancer to chemotherapeutic agents (*see e.g.*, Scher et al., 2000, "A Phase I Trial of G3139 (Genta, Inc.) a BCL2 Antisense Drug by Continuous Infusion (CI) as a Single Agent and with Weekly Taxol (T)," Proc. ASCO, 19:199a (abstract 774); Tolcher et al., 2000, "A phase I, pharmacokinetic and biologic correlative study of G3139 (Bcl-2 antisense oligonucleotide) and Docetaxel in patients with hormone-refractory prostate cancer (HRPC)," Clin Cancer Res Supp, 6:4571s (abstract 527); Marcucci et al., 2000, "Phase I Trial of Gentasense (G3139, Genta, Inc.), a bcl-2 Antisense (AS), in Refractory (REF) or Relapsed (REL) Acute Leukemia (AL)," J Amer Soc. Hematology, Blood Supp, 96(11):119a (abstract 513); and Jansen et al., 2000, "Chemosensitisation of malignant melanoma by BCL2 antisense therapy," Lancet, 356:1728-32, attached hereto as exhibits B, C, D and E, respectively. The *in vitro* efficacy of oligonucleotides targeted to strategic sites of the bcl-2 gene as defined by the claimed invention correlates with *in vivo* efficacy as demonstrated by Waters et al. (2000, "Phase I clinical and pharmacokinetic study of bcl-2 antisense therapy in patients with non-Hodgkin's lymphoma," J Clin Oncol 18:1812-23). The authors make clear that success of *in vitro* experiments provided the rationale for a phase I trial of the antisense oligonucleotide in cancer patients. The results indicate that the oligonucleotide is well absorbed by the subcutaneous route and that it reduces levels of bcl-2 in cancer patients, which correlates with a better prognosis.

The Examiner maintains that "in the instant case the specification has neither clearly defined the desired functional relationship such that the specific desired result of the antisense treatment in vivo may be clearly understood" (February 8, 2002 Office Action at page 5). Applicant respectfully disagrees and asserts that the instant disclosure adequately defines the

desired functional relationship, and thus fulfills the enablement requirement. *See Wilden Pump & Eng'r Co. v. Pressed & Welded Prod. Co.*, 199 U.S.P.Q. 390 (N.D. Cal. 1978), *aff'd*, 655 F.2d 984, 213 U.S.P.Q. 282 (9th Cir. 1981), *on remand*, 570 F.Supp. 224, 224 U.S.P.Q. 1074 (N.D. Cal. 1983) ("A patent's disclosure is adequate if it defines the desired functional relationship, even if some experimentation is required to reproduce the invention."). As stated above, the design and use of specific bcl-2 antisense oligonucleotides targeted to strategic sites for the treatment of cancer are explicitly set forth in the original specification. Moreover, there is a clear link between hybridization of the claimed oligonucleotides to strategic sites of SEQ ID NO: 19 and a concomitant decrease in bcl-2. Thus, in light of the teachings of the specification and the art, one would fully expect the claimed oligonucleotides to be effective in the treatment of cancer in humans.

Nevertheless, as previously noted by Applicant, a considerable amount of experimentation is permissible, so long as it is merely routine. *See In re Wands,* 858 F.2d 731, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Applicant reasserts that the claimed invention is fully enabled, and should not be precluded by the necessity for some experimentation involving routine screening and testing. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367 (Fed. Cir. 1986). Applicant respectfully submits that the expectation of success for identifying useful bcl-2 antisense oligonucleotides of 10 to 40 bases in length targeted against the strategic sites of SEQ ID NO:19 is no less than testing an array of hundreds (or thousands) of hybridomas to identify one useful monoclonal antibody, and thus the claimed invention is clearly within the purview of the skilled artisan. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367 (Fed. Cir. 1986). In fact, the instant technology often permits the identification of several antisense sequences involving screening of only a few dozen candidate sequences.

The Examiner further states the "in a complex whole organism such as a human, the

lack of understanding of all the physiological pathways and cause and effect of administration of a novel drug candidate precludes the expectation that any potential drug candidate will function in a specific pattern that solves the problem disease. In light of this complexity of the whole organism . . . there would be an undue amount of experimentation required to make and use the invention" (February 8, 2002 Office Action at page 5). Applicant wishes to point out that this is not the standard for enablement. The Examiner's proposed standard would preclude every drug candidate for human use in the absence of, at least, preliminary clinical data. Applicant emphasizes, however, that pharmacological data from clinical trials (or even animal models) are not required to satisfy 35 U.S.C. § 112, first paragraph. *See* M.P.E.P. §§ 2164.01(c) and 2107.03. As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. § 112 is satisfied. *In re Fisher*, 427 F.2d 833, 839, 166 U.S.P.Q. 18, 24 (C.C.P.A. 1970); *see also* M.P.E.P. § 2164.01(b).

According to the M.P.E.P., "the courts have repeatedly held, all that is required is a *reasonable* correlation between the activity and the asserted use." § 2107.03 (I) (emphasis added). Applicant asserts that the instant specification provides objective scientific data demonstrating antisense activity that *reasonably* correlates to *in vivo* applications. In fact, such assays are routinely used in the art of antisense technology to identify and test antisense oligonucleotides for human treatment. Using the methods of the instant invention, the post-filing art confirms this correlation. Thus, the teachings of the instant specification, in combination with the state of the art as of the filing date, fully enable the claimed invention.

In summary, the new claims are drawn to the treatment of cancer in humans, and the claimed oligonucleotides now target "strategic sites" of SEQ ID NO:19, which are fully described by the specification. These strategic sites are identified by the specification and the post-filing art as effective targets to decrease the level of bcl-2 and kill human cancer cells.

Since the art at the time of filing provides, *inter alia*, methods of administration, dosing and toxicity assays, and the post-art documents human treatment using pharmaceutical compositions comprising antisense oligonucleotides of the instant invention, the invention is fully enabled under 35 U.S.C. § 112. As such, Applicant respectfully requests that the rejections under 35 U.S.C. §112, first paragraph be reconsidered and withdrawn.

CONCLUSION

Applicant respectfully requests entry of the foregoing amendments and remarks into the file of the above-identified application. Applicant believes that each ground for rejection or objection has been overcome or obviated, and that all of the pending claims are in condition for allowance. Applicants respectfully request consideration of the pending claims and withdrawal of the rejection. An early allowance is earnestly sought.

Respectfully submitted, by: Jacqueline Benn
Reg No. 43,492

Date: August 8, 2002

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Enclosures

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A Phase I Trial of G3139 (Genta, Inc.), a BCL2 Antisense Drug, by Continuous Infusion (CI) as a Single Agent and with Weekly Taxol (T). *Howard I. Scher, Michael J. Morris, William P. Tong, Carlos Cordon-Cardo, Marija Drobnjak, William K. Kelly, Susan F. Slovin, Kathryn L. Terry, Robert S. DiPaola, Mohmed Rafi, Neal Rosen, Memorial Sloan-Kettering Cancer Ctr, New York, NY; Cancer Institute of New Jersey, New Brunswick, NJ.*

G3139 is an 18-mer oligonucleotide that targets the mRNA of BCL2, a protein that inhibits apoptosis and confers treatment resistance. Antitumor effects have been observed in xenografts and man. It is also synergistic with taxanes in studies using BCL2+ xenografts. In this trial, patients (pts) received G3139 as a CI for 14-21 days in escalating doses; 27 received G3139 alone at 0.6-4.1 mg/kg/d and 8 received G3139 alone for 1 cycle at 4.1, 5.3, and 6.9 mg/kg/d followed by G3139 plus T at 100 mg/m^2 weekly x 3 for 2 cycles. Pharmacokinetic (PK) profiles and BCL2 protein expression in peripheral blood lymphocytes (PBLs) were assessed. 35 pts have been treated: 23 prostate, 4 renal, 3 prostate and renal, 1 unknown primary, 1 sarcoma, 1 esophageal, 1 bladder, and 1 rectal. 2 pts (6%) had port-related clots or infections. Drug-related grade 3 adverse events were leukopenia, fatigue, and rash (1 pt, 3%, each). There were no grade 4 drug-related events. Additional events noted with T were grade 1-2 mucositis (2 pts, 6%), leukopenia (3 pts, 9%), thrombocytopenia (2 pts, 6%), abnormal LFTs (3 pts, 9%), and hyperglycemia (4 pts, 11%). 2 of 3 pts in the 6.9 mg/kg/d cohort developed grade 1-2 transaminitis on day 14, at full and again at modified doses. The $T_{1/2}$ was 2 hours, while the C_{ss} was 3-4, 4-5, and 7-8 μg/ml for doses of 4.1, 5.3, and 6.9 mg/kg/d, respectively, all exceeding the concentration (1 μg/ml) associated with response in xenografts. Western blots of PBLs in pts treated at 4.1 mg/kg/d reveal that BCL2 protein expression decreased within one week, with the peak effect seen at 8-15 days. 1 pt treated at 6.9 mg/kg/d had a radiographic and clinical response to treatment, and 1 had relief of pain, while 3 pts with renal cell had stable disease. Conclusions: G3139 is well tolerated as monotherapy and in combination with full-dose weekly T. Reversible transaminitis is anticipated to be dose limiting. G3139 can decrease BCL2 protein expression. Further study is ongoing to establish whether the time needed to achieve the peak effect on protein expression decreases at higher doses. Antitumor effects have been observed. Supported by Genta, Inc., CaPCURE, CA05826, CA09207.

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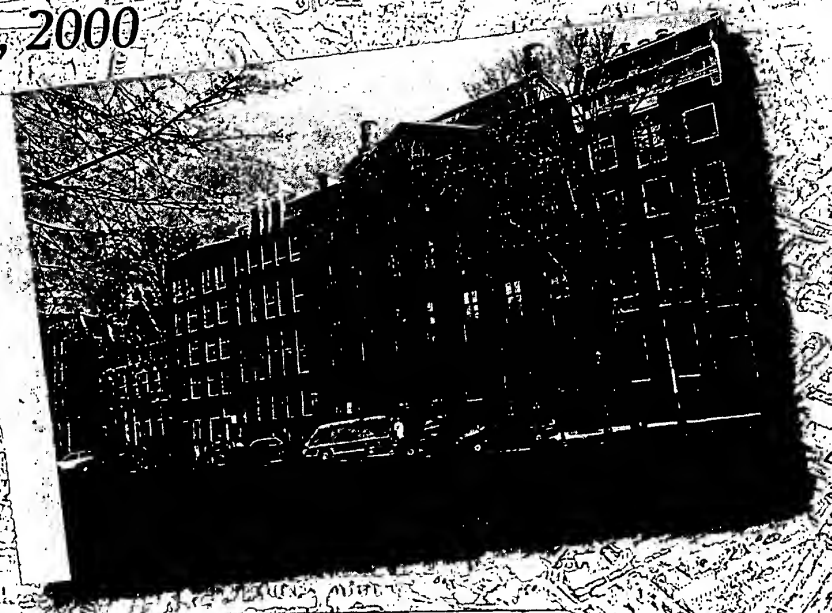

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523 Antisense oligonucleotides against the androgen receptor selectively inhibit proliferation of hormone-sensitive LNCaP cells *in vitro* and *in vivo*. Dieter Zopf[1], Dave Ayers[2], Jens Hoffmann[1], Iris Eder[3] and Jim Thompson[2]. [1]*Schering AG Berlin, Department of Experimental Oncology;* [2]*Atugen Inc., USA;* [3]*University of Innsbruck, Department of Urology.*

Androgen ablation is currently the only effective therapy of disseminated PCa. However, the duration of the response to this therapy is only 12–18 months and new avenues of therapeutic interventions are required. Therapy resistance is not due to the loss of androgen receptor (AR). Genetic changes in advanced primary and hormone-resistant PCa lead to AR-gene amplification or to AR mutations which cause AR activation by antiandrogens. Furthermore AR can be activated by residual androgens in androgen hypersensitive tumors or ligand independently by growth factor mediated signal transduction pathways. Conceptually, the elimination of AR should therefore provide a useful strategy for the treatment of primary and resistant PCa tumors. We have investigated the effects of antisense oligonucleotides against AR on the proliferation of LNCaP cells, a hormon-sensitive human prostate cancer line. AR specific Geneblocs were designed and synthesized by Atugen Inc. Two Geneblocs (15331 and 15332) selectively inhibit the proliferation of LNCaP cells after transfection. mRNA and protein levels of AR are significantly reduced. Both Geneblocs do not inhibit proliferation of AR-negative human prostate cells (PC3), non-prostate cancer cells (MCF7) or non-cancer primary cells (MRC5; CHANG). A Genebloc directed against the polyGLN-repeat of AR (15340) is a potent, but unspecific proliferation inhibitor, probably because it affects multiple polyGLN-repeat containing targets. In a PCa xenograft mouse model a phosphorothioate oligonucleotide directed against the polyGLN-repeat selectively inhibited tumor growth in mice.

524 Rational targeting of c-*myb* RNA molecules with oligodeoxynucleotides and RNA-cleaving DNAzymes. Gifford LK, Rodriguez LC, Rizzo J, Zhang X, Gewirtz AM, Lu P. *Departments of Chemistry and Medicine, University of Pennsylvania and University of Pennsylvania School of Medicine, Philadelphia, PA.*

Selective intervention in gene expression may be accomplished by targeting reverse complementary ODNs to an mRNA of interest, which results in destruction of the mRNA or interference with translation. This deceptively general approach is complicated by mRNA folding in cells, which is complex and subject to modification by associated proteins. To address this critical problem, we have synthesized reporter oligodeoxynucleotides (ODNs) called molecular beacons (MB) that are composed of a 5–7 base stem structure with an 18–20 base loop that hybridizes to the target mRNA. An algorithm was designed to develop MBs that would automatically involve the stem portion when hybridizing to the c-*myb* RNA transcripts by identifying palindromic sequences that were separated by 18 to 20 nucleotides. When these MBs were targeted to specific areas of the *in vitro* transcribed RNA, we found that out of five beacons tested, one showed 50% of maximum fluorescence, while the control sequence showed ~10% fluorescence and the other sequences exhibited 15–30% maximal fluorescence. This MB has been shown to inhibit c-*myb* expression in the hamster fibroblast cell line TK-ts113 by 70%. Another algorithm was developed to calculate the free energy of binding for the arms of a DNAzyme with target sequences in the c-*myb* transcript. A MB was developed from the sequence of one of these DNAzymes that exhibited 70% of maximum fluorescence when incubated with mRNA *in vitro*. These DNAzymes cleave synthetic targets corresponding to those found in the c-*myb* gene. In addition, we have surveyed the secondary structure conformations in a c-*myb* RNA with DNA enzymes. These *in vitro* data in the absence of protein serve as a guide for our *in vivo* experiments and underscore the necessity for a rational approach to antisense molecule design.

525 Inhibition of cell cycle progression and induction of apoptosis in leukemia cells by *Mycobacterium phlei* DNA and derived synthetic oligonucleotides. M.C. Filion, S. Reader, B. Filion, S. Ménard and N.C. Phillips. *Bioniche Therapeutics Research Centre, Montréal, Québec, Canada.*

Prokaryotic DNA and synthetic phosphorothioate CpG oligonucleotides have been shown to have anti-cancer activity against solid tumors by stimulating the immune system. However, they can prevent apoptosis and induce proliferation of leukemia cells. Recent studies from our laboratory have shown that DNA isolated from *Mycobacterium phlei* (*M. phlei*) is able to inhibit the proliferation of bladder cancer cells through the induction of apoptosis. In this report we have evaluated the anti-proliferative and apoptosis-inducing activity of *M. phlei*-derived DNA and two synthetic phosphodiester oligonucleotides (ODN) derived from *M. phlei* DNA containing either GpT dinucleotides within a specific sequence context (33 base length) or ApC dinucleotides within a specific sequence context (15 base length). *M. phlei*-derived DNA in the concentration range 1.0 to 100 μg/ml cause a dose-dependent inhibition of human Jurkat, K562, THP-1, HL-60 and HL-60 MX-1 cell division that was associated with cell cycle arrest in the late S-phase/G_2M of the cell cycle. The *M. phlei*-derived ODN containing a GpT dinucleotide motif also had the ability to block the cell cycle, but at the G_0/G_1/early S-phase. The *M. phlei*-derived ODN containing an ApC dinucleotide motif did not inhibit leukemia cell division. Inhibition of cell division was accompanied by the translocation of phosphatidyl serine to the cell surface, mitochondrial membrane potential disruption, activation of caspase-3, cleavage of poly(ADP)ribose polymerase and the release of soluble nuclear mitotic apparatus protein, characteristics of cells undergoing apoptosis. The presence of multidrug resistance or p53 mutations did not affect the ability of *M. phlei*-derived DNA or GpT-ODN to induce apoptosis. Furthermore, the induction of apoptosis was found to be independent of Fas (CD95) signalling. Our data show that *M. phlei*-derived DNA and ODN containing a GpT dinucleotide motifs possess the ability to induce apoptosis in leukemia cells but that they cause cell cycle arrest at different phases of the cell cycle.

526 Identification of non-antisense phosphodiester oligonucleotides that induce cell cycle arrest and apoptosis in cancer cells. S. Reader, B. Filion, S. Ménard, M.C. Filion and N.C. Phillips. *Bioniche Therapeutics Research Centre, Montréal, Québec, Canada.*

Antisense oligonucleotides have been developed by other groups as inhibitors of cell cycle progression and inducers of apoptosis in cancer cells. We have tested a novel series of non-antisense phosphodiester oligonucleotides (ODN) containing GpT dinucleotide motifs within specific sequence contexts for their ability to inhibit cancer cell division and to induce apoptosis. We have found that these ODN in the concentration range 1 to 100 ug/ml cause a dose-dependent inhibition of human breast, bladder, leukemia, ovarian and prostate cancer cell division that is associated with an arrest in cell cycle progression. We have identified three distinct families of ODN with the ability to induce cell cycle arrest after 24 h of incubation either in 1) G_0/G_1/early S-phase, 2) middle S-phase or 3) late S-phase/G_2/M. The optimal ODN length is 6 bases. Among these ODN, we have identified a potent 6 base-length ODN containing a GpT dinucleotide motif with the capacity to effectively block cell division at the G_0/G_1/early S-phase of the cell cycle and to induce apoptosis, as characterized by the translocation of phosphatidyl serine at the cell surface, the cleavage of poly-(ADP)ribose polymerase and the release of soluble nuclear mitotic apparatus protein. The presence of atypical and typical multidrug resistance or of p53 and/or p21/waf-1 mutations did not affect the ability of this ODN to induce apoptosis. Substitution of the phosphodiester linkage by its phosphorothioate analog, which is known to increase the stability of ODN against nuclease digestion, was found to abolish its activity. The induction of cell cycle arrest and apoptosis by this ODN was also inhibited by hybridization to its complementary sequence, suggesting that this activity is both sequence specific and associated with single stranded DNA. In summary, we have identified a novel class of molecules capable of modulating the cell cycle of cancer cells and of inducing apoptosis. These non-antisense phosphodiester ODN may have potential as chemotherapeutics.

527 A phase I, pharmacokinetic and biologic correlative study of G3139 (Bcl-2 antisense oligonucleotide) and Docetaxel in patients with hormone-refractory prostate cancer (HRPC). AW Tolcher[1,2], J Kuhn[1], J Basler[2], L Ochoa[1], G Schwartz[3], A Patnaik[1], L Hammond[1], L Smetzer[1], L Smith[1], H Fingert[4], S. Weitman[1], I Thompson[2], EK Rowinsky[1]. [1]*Institute for Drug Development, CTRC, San Antonio, TX,* [2] *Division of Urology, UTHSCSA,* [3]*Brooke Army Medical Center, San Antonio TX,* [4] *Genta Inc, Lexington, MA.*

G3139 is an antisense oligonucleotide directed to the first 6 codons of the *bcl-2* mRNA. Bcl-2 protein is overexpressed in the majority of patients with HRPC and confers resistance to both androgen ablation therapy and cytotoxic chemotherapy. In mice bearing human prostate cancer (LNCaP & PC3) xenografts, G3139 led to degradation of bcl-2 mRNA and downregulation of Bcl-2 protein, and markedly enhanced docetaxel chemosensitivity including cures in nude mice bearing PC3 xenografts. In this phase I study, G3139 is administered as a continuous intravenous infusion days 1–6, followed by docetaxel administered IV on day 6. Courses are repeated every 21 days. To date, 11 pts with HRPC have received 28 courses of therapy at 3 dose levels ranging from G3139 at 5 mg/kg/day with docetaxel 60 mg/m^2, to G3139 at 7 mg/kg/day docetaxel 75 mg/m^2. One heavily pretreated patient had prolonged (> 5 days) uncomplicated grade 4 neutropenia which has led to 6 patients being accrued the latter dose level. Other toxicities related to docetaxel and G3139 include stomatitis (grade 1) in 3 pts, and febrile neutropenia during course 2 in 1pt. Preliminary pharmacokinetic results (HPLC) demonstrate a mean (±SD) G3139 steady state plasma concentrations of 3.09 μg/mL at 5 mg/kg/day G3139 dose level. Preliminary flow cytometric and western blot analysis indicate marked downregulation of Bcl-2 protein by day 6 in peripheral blood mononuclear cells (MNC). PSA responses have been seen in 2 of 3 evaluable taxane naïve patients including a 9-fold reduction in PSA. G3139 can be safely administered in combination with docetaxel, effectively downregulates Bcl-2 protein in MNC, and has encouraging preliminary antitumor activity in HRPC patients.

* Study Supported by a grant from Aventis Pharmaceuticals.

528 A phase I and pharmacokinetic (PK) study of MG98, a human DNA methyltransferase (MeTase) antisense oligonucleotide, given as a 2-hour twice weekly (BIW) infusion 3 out of every 4 wks. D. Stewart[1], R. Donehower[2], E. Eisenhauer[3], N. Wainman[3], M. Moore[4], C. Bonfils[5], G. Reid[5]. [1]*Ottawa Regional Cancer Centre;* [2]*Johns Hopkins Oncology Center;* [3]*NCIC Clinical Trials Group;* [4]*Princess Margaret Hospital,* [5]*MethylGene Inc., Montreal, Canada.*

Hypermethylation by DNA MeTase is postulated to inactivate tumor suppressor genes leading to neoplastic transformation. Thus inhibition of MeTase might restore normal growth control. MG98, a 2nd generation phosphorothioate antisense oligodeoxynucleotide, is a specific inhibitor of the mRNA for human DNA MeTase with IC_{50} values of 50–70 nM in cell lines. In xenograft models it led to tumor growth delay and regression. In this phase I study MG98 is administered as a 2-hr IV infusion biw, 3 of every 4 weeks. To date, 11 pts with solid cancers (M/F 10:1, median age 69 yrs, median PS ECOG 1) have received

blood

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and CD80/CD40L/GMCSF despite different levels of protection. Similarly, immunologic memory responses were roughly equivalent between CD80/GMCSF and CD80/CD40L/GMCSF with 57% and 39% long term survivors respectively after re-challenge with wild type BM185 cells. This low memory response may explain the lower numbers of survivors in the vaccination setting when compared to live challenges. We are currently comparing CD80/GMCSF and CD80/CD40L/GMCSF vaccinations for their ability to eradicate minimal residual disease in this model. We hypothesize that CD40L activation of NK cells will keep the tumor burden low until an adequate CTL and memory response is initiated by CD80 and GMCSF. These studies illustrate the complex interactions among immunomodulator genes; the ability of CD40L to act in a T cell-independent manner may be important for clinical applications in patients with depressed cellular immunity following chemotherapy.

Abstract# 511 **Poster Board #-Session: 511-I**

HISTONE DEACETYLASE INHIBITORS UP-REGULATE COSTIMULATORY/ADHESION MOLECULES IN ACUTE MYELOID LEUKEMIA CELLS. Takahiro Maeda*,[1] Masayuki Towatari,[1] Hiroshi Kosugi*,[1] Hidehiko Saito.[1] *[1]1st Internal Medecine, Nagoya University School of Medecine, Nagoya, Japan.*

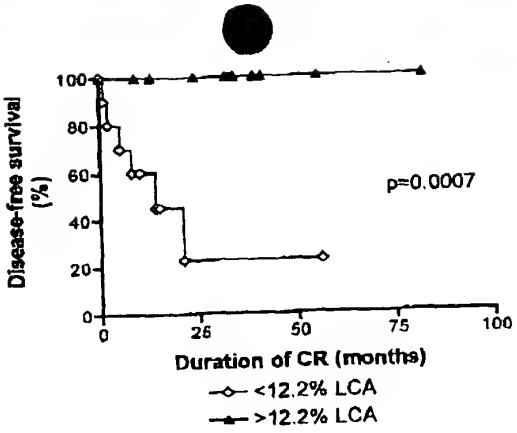
Histone deacetylase Inhibitors (HDACIs) are thought to inhibit the enzymatic activity of histone deacetylase, and to enhance the transcriptional activity of several genes. Modulation of immune responses through acetylation and deacetylation is poorly understood. We hypothesized that acetylation and deacetylation are among the major mechanisms in the regulation of costimulatory/adhesion molecules.We explored whether HDACIs could induce the expression of costimulatory/adhesion molecules on acute myeloid leukemia (AML) cells, thereby effectively inducing tumor immunity. We first tested the expression of CD80, CD86, HLA-DR, HLA-ABC and ICAM-1 after the addition of HDACI, sodium butyrate (SB), in human AML cell lines. Generally, increased expression of CD86 was observed by SB treatment in a majority of cell lines, and that of ICAM-1 was expressed in a fewer cell lines examined. Essentially the same results were obtained by the other HDACIs, FR901228, trichostatin A, and trapoxin A. Quantitation of transcripts of CD86 in accompany with RNA synthesis inhibition assay and nuclear run-on assay revealed that SB up-regulates the CD86 expression transcriptionally. Furthermore, chromatin immunoprecipitation (ChIP) experiments showed HDACI treatment caused remarkable acetylation on histone H3 and H4 at CD86 promoter chromatin in vivo. In thirty clinical AML samples, CD86 expression was significantly increased ($P<0.001$) and that of HLA-DR and ICAM-1 was moderately increased ($P<0.05$) by SB treatment. Finally, the allogeneic mixed leukocyte reaction (allo-MLR) against HL60 cells pretreated with SB was enhanced 4-fold over that obtained with non-treated HL60 cells. These results suggest that the immunotherapeutic use of HDACIs may become a novel tool for treatment of AML.

Abstract# 512 **Poster Board #-Session: 512-I**

AUTOLOGOUS LEUKAEMIA-REACTIVE CD8+ NK CELLS ARE REQUIRED FOR LONG-TERM SURVIVAL AFTER TREATMENT FOR ACUTE LEUKAEMIA. Mark W. Lowdell*,[1] Marion E. Wood*,[2] David Samuel*,[3] Rose Craston*,[1] Vaska Saha*,[3] H. Grant Prentice.[1] *[1]Haematology - RF Campus, Royal Free & University College Medical School, London, United Kingdom; [2]Haematology, Colchester General Hospital, Colchester, Essex, United Kingdom; [3]ICRF Childrens' Cancer Group, Royal London Hospital, London, United Kingdom.*

Graft-versus-leukaemia after allogeneic BMT is known to be mediated by immune mechanisms. We have shown that immune-mediated anti-leukaemia activity is the mechanism of cure after autologous BMT. To further explore this phenomenon, we have studied 18 patients after chemotherapy alone and a further five patients after autologous BMT (aBMT) conditioned with radio/chemotherapy. All patients were undergoing treatment for acute leukaemia (AML - 12; ALL -11) and the group consisted of 15 adults and 8 children (all cALL). Lymphocytes from each patient were collected in remission at completion of therapy and tested for in vitro cytolytic activity against their own presentation leukaemic blasts. Patients who subsequently relapsed were found to have significantly lower leukaemia-cytolytic activity (LCA) than those who remain in remission beyond two years ($p<0.001$) and the absence of LCA activity was able to predict relapse with a sensitivity of 100% and specificity of 91% (FIG 1). The subset of lymphocytes mediating this activity in vitro were CD56+/CD8wk+/CD3- natural killer cells (NK) and the cytolytic signal was mediated through CD8 and CD69 molecules. Patients who achieve remission after chemotherapy, with or without subsequent autologous BMT may develop protective levels of NK-mediated LCA and there is a strong correlation between the presence of this activity and long-term maintenance of remission. Therefore, NK cells may be responsible for control of the disease in these settings.

Figure 1:



Abstract# 513 **Poster Board #-Session: 513-I**

PHASE I TRIAL OF GENASENSE™ (G3139, GENTA, INC.), A BCL-2 ANTISENSE (AS), IN REFRACTORY (REF) OR RELAPSED (REL) ACUTE LEUKEMIA (AL). G. Marcucci,[1] C.D. Bloomfield,[1] S.P. Balcerzak,[1] P.J. Kourlas*,[1] H.R. Stanley*,[1] H. Fingert*,[2] E.A. Maghraby*,[1] D. Lucas*,[1] K.K. Chen*,[1] J.C. Byrd,[1] E.H. Kraut,[1] M.R. Grever,[1] M.A. Caligiuri.[1] *[1]The Comprehensive Cancer Center, The Ohio State University, Columbus, OH; [2]GENTA, INC, Lexington, MA.*

In AL a strong association between chemotherapy resistance and overexpression of BCL-2 exists. We hypothesized that chemotherapy-induced apoptosis is enhanced by BCL-2 downregulation. G3139 is a BCL-2 AS that downregulates BCL-2 expression in vitro and in vivo. We report on 10 pts enrolled at levels 1-3 of a Phase I study with G3139 + fludarabine, ARA-C, and G-CSF (FLAG) therapy for REF/REL AL. G3139 (4mg/kg/day) is given on d1-10, whereas both fludarabine (starting @15mg/m2) and ARA-C (starting @1000mg/m2) are given on d6-10 and escalated in successive cohorts.

Pts age/sex	Dx & Status pre-G3139	Time to REL(m)[1]	Previous Regimens	Previous HDAC[2]	Response	Disease status (d)[3]
69/F	primary REF ALL	NA[4]	1	No	CR[5]	NED[6] (53)
55/F	primary REF AML	NA	3	Yes	CR	REL (142)
57/F	2nd REL AML	12	2	Yes	CR	NED (111)
23/M	1st REL AML	3	1	Yes	PR[6]	REL (83)
61/F	1st REL AML	7	1	No	PR	NED (76)
54/M	primary REF AML	NA	1	No	NR[7]	REF
61/F	1st REL AML	6	2	No	NR	REF
73/F	2nd REL AML	8	2	Yes	NR	REF
39/M	2nd REL AML	3	2	Yes	NR	REF
55/F	2nd REL AML	6	3	Yes	NR	REF

[1](m), months from CR; [2]high-dose ARA-C; [3](d), days from G3139 start; [4]NA,not applicable; [5]CR, complete response; [6]PR, partial response;[7]NR, no response;[6]NED, no evidence of disease.

Therapy-related fever, nausea, emesis, hypocalcemia, hypophosphatemia, and fluid retention were not dose-limiting. Hematologic toxicities were as expected. Steady state G3139 plasma levels exceeding the relevant target level (1ug/ml) were achieved after 24h. Quantification of BCL-2 levels in AL blasts will be presented. Three pts achieved CR and received a 2nd course of therapy, two continue with NED at d53 and 111. Two pts had NED but persistent neutropenia/thrombocytopenia at d52 and 55; one of them continues with NED at d76. Three of 5 responders had prior HDAC. One patient had leukostasis at d6, and was taken off study. The data suggest that G3139 is feasible for addition to multi-cycle induction regimens for AL; moreover the encouraging 50% response rate - including pts with REF AL and prior HDAC - supports further development of G3139 in combination regimens for REL/REF AL.

Early report

Chemosensitisation of malignant melanoma by *BCL2* antisense therapy

B Jansen, V Wacheck, E Heere-Ress, H Schlagbauer-Wadl, C Hoeller, T Lucas, M Hoermann, U Hollenstein, K Wolff, H Pehamberger

Summary

Background Chemoresistance of malignant melanoma has been linked to expression of the proto-oncogene *BCL2*. Antisense oligonucleotides (ASO) targeted against *BCL2* mRNA decreased BCL2 protein concentrations, increased tumour-cell apoptosis, and led to tumour responses in a mouse xenotransplantation model when combined with systemic dacarbazine. This phase I–II clinical study investigated the combination of *BCL2* ASO (augmerosen, Genasense, G3139) and dacarbazine in patients with advanced malignant melanoma expressing BCL2.

Methods In a within-patient dose-escalation protocol, 14 patients with advanced malignant melanoma were given augmerosen intravenously or subcutaneously in daily doses of 0·6–6·5 mg/kg plus standard dacarbazine treatment (total doses up to 1000 mg/m² per cycle). Toxicity was scored by common toxicity criteria. Plasma augmerosen concentrations were assayed by high-performance liquid chromatography. In serial tumour biopsy samples, BCL2 protein concentrations were measured by western blotting, and tumour-cell apoptosis was assessed.

Findings The combination regimen was well tolerated, with no dose-limiting toxicity. Haematological abnormalities were mild to moderate. Lymphopenia was common, but no febrile neutropenia occurred. Higher doses of augmerosen were associated with transient fever. Four patients had liver-function abnormalities that resolved within 1 week. Steady-state plasma concentrations of augmerosen were attained within 24 h, and increased with administered dose. By day 5, daily doses of 1·7 mg/kg and higher led to a median 40% decrease in BCL2 protein in melanoma samples compared with baseline, concomitantly with increased tumour-cell apoptosis, which was greatly increased after dacarbazine treatment. Six patients have shown antitumour responses (one complete, two partial, three minor). The estimated median survival of all patients now exceeds 12 months.

Interpretation Systemic administration of augmerosen downregulated the target BCL2 protein in metastatic cancer. Such downregulation of BCL2, combined with standard anticancer therapy, offers a new approach to the treatment of patients with resistant neoplasms.

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Introduction

Localised melanoma has a good prognosis after adequate surgical therapy, but the prognosis of metastatic melanoma is poor despite the availability of a growing number of biological, chemotherapeutic, and combined-modality treatments. Regimens including dacarbazine remain the most commonly used for metastatic melanoma, but few patients obtain durable responses, and the long-term survival benefit from this drug or the related orally administered agent temozolomide is limited.[1] A large phase II clinical trial did not demonstrate survival benefit of a widely used combination regimen (dacarbazine, cisplatin, carmustine, tamoxifen) compared with single-agent dacarbazine treatment.[2] Thus, new treatment approaches are needed.

Antisense oligonucleotides are chemically synthesised, highly purified sequences of single-stranded DNA that are complementary to specific coding regions of mRNA. They can inhibit gene expression in various tumour systems, including melanoma.[3] The antisense drug augmerosen (Genasense, Genta Inc, USA; code G3139) an 18-base phosphorothioate oligonucleotide, complementary (antisense) to the first six codons of *BCL2* mRNA hybridises to the respective target RNA bases, which leads to selective decreases in concentrations of the RNA and protein product. Laboratory and clinical evidence suggests that BCL2 protein can exist in a delicate balance with other related proteins to block apoptosis, one of the most common pathways for tumour-cell death produced by cytotoxic chemotherapies such as dacarbazine.[4] A role for *BCL2* in the development of normal melanocyte stem cells is suggested by the observation that *Bcl2* knock-out mice lose their dark hair colour after birth owing to rapid degeneration of hair bulb melanocytes.[5] In melanoma cells, the malignant counterparts of melanocytes, BCL2 expression is found in up to 100% of cases[6] and high BCL2 expression has been associated with shorter survival and resistance to chemotherapy in melanoma and other cancers.[4,7]

BCL2 ASO (augmerosen) was studied as a single agent in extensive, drug-resistant non-Hodgkin lymphoma[8] and was well tolerated with some objective responses.[9] In a xenotransplantation model for human melanoma, systemic treatment with augmerosen decreased concentrations of BCL2 and increased apoptosis. As a

single agent, augmerosen had slight antitumour activity, but stronger activity was observed when it was combined with dacarbazine. In ten of 13 animals, no malignant melanoma xenografts were detectable after augmerosen combined with dacarbazine treatment.[3] On the basis of these findings, we undertook a phase I–II study of combination therapy with augmerosen and dacarbazine in patients with advanced malignant melanoma, including patients with disease resistant to dacarbazine regimens and other first-line therapies.

Methods

Patients with stage IV metastatic melanoma were eligible for this dose-escalation study if they had measurable disease, and if cutaneous metastases were accessible for biopsy and initially positive for BCL2 protein by western blotting. Patients were required to have normal renal, hepatic, and haemopoietic function, and to have received no chemotherapy or immunotherapy during the preceding 4 weeks.

The patients were ambulatory during most of the augmerosen therapy, but they were admitted to hospital to facilitate pharmacokinetic monitoring and serial biopsies required in this study. Augmerosen (sequence 5′-TCTCCCAGCGTGCGCCAT-3′) was administered as a continuous intravenous infusion for 14 days by an ambulatory infusion pump (Sims Deltec Inc, St Paul, MN, USA) through a central venous line. Dacarbazine was administered through a separate peripheral intravenous line at doses of 200 mg/m² daily given by 1 h infusions on days 5 to 9 of the augmerosen course. Treatment cycles were repeated monthly. The initial dose of augmerosen was 0·6 mg/kg daily; the dose steps were (in mg/kg daily) 1·3, 1·7, 2·1, 3·1, 4·1, 5·3, and 6·5. Once safety had been established in at least three patients at a given dose, new cohorts were entered at the next higher dose.[8] Repeat 28-day cycles and dose escalation for individual patients were permitted in stable or responding patients after 2 weeks of observation.

To gain clinical experience with an alternative route and schedule, we treated six patients in the cohorts receiving 5·3 mg/kg or 6·5 mg/kg daily with one cycle by intravenous infusion then subcutaneous administration in subsequent cycles. These patients received the same total daily dose, administered by twice-daily subcutaneous injections on days 1 to 7, combined with dacarbazine 800 mg/m² given as a 1 h infusion on day 5.

Antitumour effects were assessed after every cycle of treatment, by calliper measurement and detailed photodocumentation of patients with skin metastases; visceral metastases were documented and followed up by computed tomography. For classification of tumour response, WHO criteria were used, requiring documentation of minimum response duration of 4 weeks. We defined complete response as disappearance of detectable metastases and partial response as a 50% or greater reduction in measurable metastases. For patients who had many metastases in one organ, at most five target lesions were documented at baseline and followed up for assessment of response. An increase in measurable disease of more than 25%, or the appearance of new metastatic lesions, was defined as progressive disease. In addition, a decrease in target-lesion diameters of less than 50% but more than 25% was designated as a minor response. All other courses were defined as stable disease. Survival was assessed from the time of first treatment on this protocol.

Toxicity was scored by common toxicity criteria, daily during drug administration, then weekly between cycles.

Patient	Age	Sex	Date of diagnosis	Metastases	Previous therapy
1	49	F	Aug 1995	LNN, skin	D, IFNα, RT, HEP
2	41	F	March 1995	Skin	CP, IFNα, RT, HEP
3	69	M	June 1994	LNN, skin	D, IL2, GM-CSF
4	52	M	May 1998	LNN, skin	D, IFNα
5	63	F	Jan 1992	Skin	D, IFNα
6	56	M	Aug 1996	Lung, skin	D, IFNα
7	61	F	May 1997	Lung, liver, skin	D, F
8	60	F	March 1995	Skin	IFNα, RT
9	75	F	June 1998	LNN, skin	IFNα
10	44	F	April 1986	LNN, skin	IFNα
11	63	M	April 1997	Lung, skin	IFNα, CP, CIS
12	90	F	July 1994	LNN, skin	None
13	67	M	June 1996	Lung, skin	None
14	76	M	April 1999	Lung, skin	IFNα

LNN=lymph nodes; D=dacarbazine; IFNα=interferon alfa; RT=radiotherapy; HEP=hyperthermic extremity perfusion; CP=carboplatin; IL2=interleukin 2; GM-CSF=granulocyte macrophage colony-stimulating factor; F=fotemustine.; CIS=cisplatin.

Table 1: **Characteristics of participating patients**

Any treatment-related grade 3 or 4 toxic effect that did not resolve during the 2 weeks between treatment cycles was judged to be dose limiting. Plasma samples to investigate augmerosen pharmacokinetics were collected before treatment and on days 2, 3, 5, 6, 10, and 14 in patients receiving the 2-week intravenous infusion; 12 h pharmacokinetic profiles were obtained in patients receiving augmerosen subcutaneously. Plasma concentrations were assayed by high-performance liquid chromatography (Pharmanalyt, Baden, Austria).[10]

BCL2 expression and the apoptotic rate of melanoma metastases were assessed by western blotting and the TUNEL (terminal deoxynucleotide-transferase-mediated dUTP nick end labelling) method, respectively.[3] Decreases in BCL2 expression of less than 20% compared with baseline values were not classed as significant owing to technical limitations. Biopsied tumours were selected on the basis of size, location, and clinical features to be similar to the target lesions used for measurement of response. Excision biopsies of cutaneous melanoma metastases were done at baseline and on day 5 of each augmerosen dose before administration of dacarbazine; additional samples were obtained up to cycle day 14 to document the effects of combined treatment. Two to four biopsy samples per patient per dose have been investigated. The portion of the tumour biopsy sample used for western blots and TUNEL assay was also assessed by routine histopathology to ensure consistent tumour-cell content and to limit confounding effects of non-tumour cells in biopsy sample.

The protocol was approved by the University of Vienna Ethics Committee, conducted under International Conference on Harmonisation–Good Clinical Practice guidelines, and monitored by an independent contract research organisation, Clinical Investigation Support (CIS) Incorporated, Vienna, Austria. All patients gave written informed consent.

Results

14 patients were treated with augmerosen (0·6–6·5 mg/kg daily) combined with dacarbazine (table 1). All 14 patients had tumours of stage IV. A total of 47 cycles of augmerosen plus dacarbazine have been administered.

Steady-state plasma concentrations of augmerosen were observed after 1 day of continuous intravenous infusion and increased in linear relation to the administered dose. Daily doses of 1·7 mg/kg or more led to consistent steady-state plasma concentrations over 1 mg/L, a concentration found to be bioactive in animals.[11] At 6·5 mg/kg daily, a mean steady-state plasma concentration of 6·47 mg/L (SD 2·51) was reached by 24 h. Plasma concentrations after subcutaneous bolus

Patient	Augmerosen dose (mg/kg daily)	Maximum decrease in BCL2 (%)	Response	Survival (months)
1	0·6	0	PD	6·6
2*	0·6–6·5	40	PR	20·5
3*	0·6–6·5	40	PR	23·3†
4	0·6–4·1	35	MR	13·7
5	3·1–4·1	20	MR	5·1
6	3·1–5·3	60	PD	2·4
7	4·1	20	PD	7·1
8*	5·3–6·5	60	Stable	15·3†
9*	5·3–6·5	60	MR	15·3†
10	6·5	NA	PD	14·4†
11	6·5	0	PD	12·5†
12*	6·5	70	CR	12·5†
13*	6·5	0	PD	1·1
14	6·5	40	Stable	7·8

PD=progressive disease; PR=partial response; MR=minor response; CR= complete response; NA=not applicable. *Received augmerosen subcutaneously, after initial intravenous treatment. †Observation continues.

Table 2: **Responses**

injections administered twice daily were bell-shaped over 12 h. A peak concentration of 8·60 mg/L (1·26) was observed 3–4 h after injection of the subcutaneous dose of 3·25 mg/kg administered every 12 h. For more than 90% of the 12 h between subcutaneous injections, plasma concentrations exceeded 1 mg/L. No changes in the pharmacokinetic properties were observed in patients receiving several cycles of therapy; concurrent dacarbazine treatment did not affect steady-state augmerosen plasma concentrations (data not shown).

At baseline, BCL2 protein expression of cutaneous melanoma metastases[6,12] was confirmed by western blotting in all 14 patients screened for this study. Serial biopsy samples from similar lesions showed decreases in BCL2 protein concentrations during augmerosen administration in most patients (table 2, figure 1). In patient 10, serial tumour samples could not be tested by western blotting owing to the lack of melanoma cells in the tissue sample. In patients treated by 14-day infusion that started on treatment day 1, the maximum decrease in BCL2 protein was typically observed after 4 days of infusion (treatment day 5), without further decrease on day 14 (data not shown). Ten of the 12 evaluable patients with augmerosen plasma concentrations above 1 mg/L showed a clear reduction in *BCL2* expression (table 2). Treatment cycles with augmerosen daily doses of 1·7 mg/kg or more showed a median reduction in BCL2 protein expression of 40% by day 5. In patients 11 and 13, no BCL2 downregulation was observed despite

Figure 1: **BCL2 downregulation after 5 days of augmerosen treatment in melanoma biopsy samples from patient 12**
Additional band on day 5 is consistent with appearance of actin subunits associated with tumour-cell apoptosis. BCL2 protein is 70% lower on day 5 by scanning densitometry, normalised against changes in the actin band.

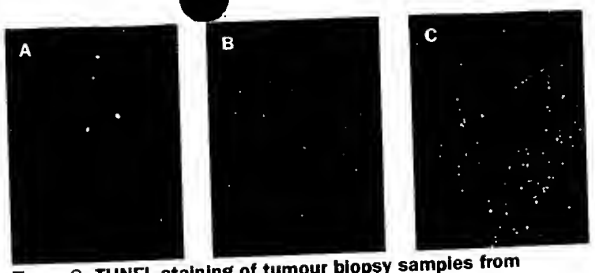

Figure 2: **TUNEL staining of tumour biopsy samples from patient 12 (right leg)**
A=before treatment; B=after augmerosen (day 5); C=after augmerosen and dacarbazine (day 14).

steady-state plasma concentrations above 1 mg/L. Both these patients experienced progressive disease. In contrast, a complete response was observed in patient 12, who showed the greatest decrease (70%) in BCL2.

We found an increase in apoptotic cells in tumour samples after 5 days of augmerosen treatment as shown by TUNEL staining (from 0·85% [SD 0·47] at baseline to 3·17% [1·16]; figure 2). However, in samples taken after addition of the apoptotic stimulus (dacarbazine), there was a further large increase in apoptotic cell death (19·4% [4·2]).

The combination therapy was well tolerated in augmerosen doses up to and including 6·5 mg/kg without dose-limiting toxicity (table 3). The only serious adverse event encountered was a catheter-related thrombosis due to incorrect positioning of the device; it was judged not to be related to augmerosen administration.

Haematological abnormalities were mild or moderate and followed the pattern of nadir values between treatment cycles typical for single-agent dacarbazine. No patient had febrile neutropenia or other major clinical haematological toxic effects. Grade 3 anaemia, requiring transfusion, occurred in two patients, but anaemia was present at baseline in these patients, possibly caused by previous treatments. Grade 2–3 lymphopenia was commonly observed, but without clinical sequelae such as unusual viral or fungal infections, or other clinical evidence of immunosuppression, despite repeat cycles and follow-up lasting over a year in some patients. Transient grade 2–3 prolongation of partial thromboplastin time was observed in three patients without clinical bleeding.

Augmerosen doses of more than 4·1 mg/kg daily were associated with transient fever in most patients. The

Event	Number of patients with event of common toxicity criteria grade			
	0	1	2	3
Haematological				
Anaemia	12	..	..	2
Leucopenia	7	2	3	2
Neutropenia	10	2	2	..
Lymphopenia	1	1	7	5
Thrombocytopenia	8	4	2	..
Coagulation	3	8	2	1
Non-haematological				
Cardiovascular	14	..	..	..
Pulmonary	14	..	..	..
Renal	14	..	..	..
Gastrointestinal	9	5	..	..
Liver function*	1	7	2	4
Neurological (headache)	11	3	..	..
Dermatological	9	4	1	..
Fever	7	1	6	..

Events are listed irrespective of causal relation to therapy. No grade 4 events occurred. *Aspartate aminotransferase, bilirubin.

Table 3: **Adverse events during treatment**

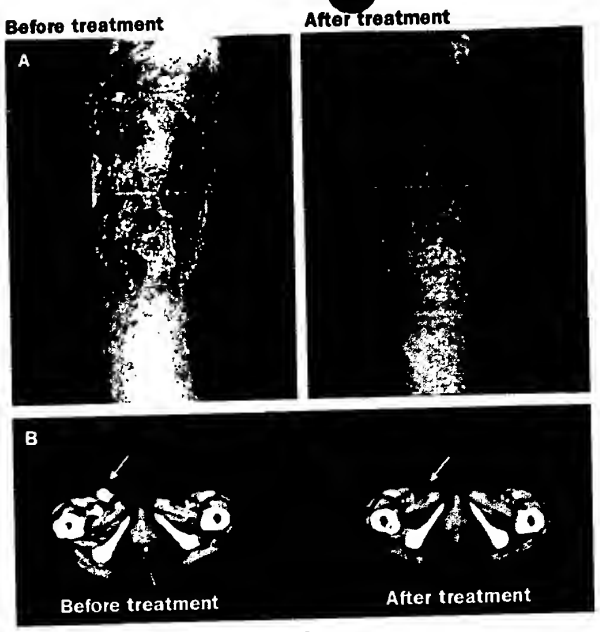

Figure 3: **Response in patient 12**
A=skin metastases; B=computed tomography of pelvis.

temperature commonly reached 38°C on days 2–3 of therapy and resolved spontaneously or with paracetamol and continued augmerosen administration. At doses of 4·1–6·5 mg/kg daily, transient grade 3 rises in aspartate aminotransferase activity or bilirubin concentration occurred in four patients; however, the causal relation to augmerosen was not established, because one patient had hepatitis and one alcoholism, and the transient liver-function abnormalities were observed after dacarbazine, which can also lead to such laboratory changes. The liver-function abnormalities typically resolved in 1 week between treatment cycles and were not judged clinically significant or dose limiting. Dermatological adverse events included transient rashes and urticaria, grade 1 in all but one patient; these reactions responded to antihistamines and did not prevent subsequent therapy. No cumulative toxic effects were observed. Some patients were treated with up to ten cycles of therapy without requiring modifications of the planned treatment schedules.

Although toxicity was the main endpoint of this dose-escalation trial, antitumour activity was evident in six of the 14 patients, some of whom had previously received systemic melanoma treatments without success (tables 1 and 2). One complete response, two partial responses, and two minor responses with stabilisation of disease for at least 1 year were noted. Clinical antitumour activity was also seen in two patients with stabilisation of disease that had been progressing before the study. Patient 12, who had bulky metastatic disease measuring over 5 cm at baseline in pelvic lymph nodes and at the site of a previous skin graft, showed a first response after two cycles and complete response after four cycles (figure 3). After four cycles of therapy, a biopsy of the cutaneous area that had been previously positive for melanoma showed only fibrosis (pathological complete response). Patients 2 and 3 showed partial response of target lesions with progression-free survival lasting over 1 year. At entry to the study, these patients had progressive metastases despite previous treatment (table 1). Patients 5 and 9 entered the study with progressive metastatic disease despite systemic therapy and showed minor responses. In all patients the estimated median survival to date exceeds 1 year. Follow-up continues.

Discussion

The primary aim of this study was to assess the toxicity of augmerosen combined with full-dose dacarbazine therapy. Several side-effects have been described for phosphorothioate oligonucleotides, which are primarily related to the backbone chemistry.[13,14] Well-known side-effects of dacarbazine include myelosuppression, nausea, and influenza-like syndromes consisting of fever, myalgia, and malaise. Flushing and urticaria have also been observed. For augmerosen doses up to and including 3·1 mg/kg daily, no non-haematological side-effects other than those reported for single-agent dacarbazine therapy were noted in this study. With doses of 4·1 mg/kg daily and higher, there were transient liver-function abnormalities, as previously reported with single-agent augmerosen.[8,9] In our study, the liver-function abnormalities were not dose-limiting or associated with adverse clinical sequelae, and some events may have been associated with underlying medical disorders. Non-dose-limiting changes in activated partial thromboplastin time, possibly linked to the heparin-like polyanionic nature of phosphorothioate oligonucleotides, were noted with augmerosen daily doses of 5·3 mg/kg and higher. These changes have also been described with other single-agent phosphorothioate antisense compounds.[9,13,14] The non-haematological side-effects observed, if not related to dacarbazine, are probably due to non-antisense effects linked, in a dose-dependent way, to the backbone chemistry. Moreover, some toxic effects observed in patients receiving augmerosen, such as liver-function abnormalities and thrombocytopenia, may be related to duration of infusion for longer than 1 week. Phase I results suggest that shorter infusion periods (5 days) may be better tolerated in combination with cytotoxic therapy for patients with solid tumours.[15] In follow-up to the treatment regimens investigated in this trial, augmerosen has been administered intravenously by infusion (7 mg/kg daily) over 5 days to five patients before dacarbazine 1000 mg/m^2 in each 21-day cycle, and was satisfactorily tolerated.

Initially, there was speculation that antisense-related BCL2 downregulation in normal tissues could lead to unusual or excessive toxic effects, possibly potentiated by chemotherapy. However, BCL2 expression in normal adult tissues is restricted,[16] and no unusual toxic effects have been observed in our studies of combinations with chemotherapy, or in clinical trials of single-agent *BCL2* antisense treatment.[8,9] In a study with a different antisense drug designed to downregulate *Bcl2* in mice, Miyake and colleagues found that antisense treatment selectively affected expression of the protein in a mouse model of prostate cancer without significantly affecting *Bcl2* expression or causing adverse effects in a various normal tissues and organs.[17]

Haemopoiesis is normal in *Bcl2*-deficient mice, and the *Bcl2* gene does not seem to be essential for survival of pluripotent stem cells.[18,19] *Bclx*, a homologue of *Bcl2* may be more important in the regulation of apoptosis in haemopoietic precursors,[20] because mice deficient in that gene undergo massive cell death of immature haemopoietic cells and neurons, and die at around 13 days of gestation.[21] Therefore, specific BCL2 downregulation is not expected to lead to a loss of renewal potential for normal haemopoietic cells. However, mature B and T lymphocytes seem to depend on BCL2 for survival[18] and lymophopenia has been

reported in a study evaluating single-agent *BCL2* antisense therapy in non-Hodgkin lymphoma.[8]

Lymphopenia was also the most frequent haematological side-effect noted in this study, consistent with the sequence-specific antisense mechanism; this effect has not been reported with antisense phosphorothioate drugs developed to downregulate other targets. The lymphopenia was not clinically significant, and there were no unusual infections in patients treated with cyclic therapy and followed up for 1 year. By contrast, some cases of thrombocytopenia have been observed with multiple phosphorothioate antisense drugs, and this side-effect was dose limiting in the study in non-Hodgkin lymphoma.[8] Even though our study combined augmerosen with chemotherapy, and steady-state plasma concentrations were higher than in the non-Hodgkin lymphoma study, we did not observe dose-limiting thrombocytopenia. The higher tolerable dose and plasma concentrations in our study are consistent with those reported from a study of augmerosen plus paclitaxel in patients with prostate cancer and other solid tumours.[22]

Conventional chemotherapy regimens aim to achieve optimum tumour-cell kill by administration of the maximum tolerated dose of a given drug. By contrast, treatment options including biological agents and oligonucleotides may require a regimen built on the concept of optimum biological dose. Our data show that the biologically relevant steady-state plasma concentration (>1 mg/L) can be easily achieved with augmerosen doses of about 2 mg/kg daily, and the maximum tolerated dose has not been reached in combination with dacarbazine chemotherapy.

Preclinical models have shown that augmerosen can specifically downregulate BCL2 protein in human melanoma xenografts, an effect not observed with reverse control or two-base-mismatch control oligonucleotides.[3] Here, similar decreases in BCL2 concentrations occurred in the setting of clinical cancer therapy, serially measured in samples of cutaneous melanoma metastases. As in mice, the changes in Bcl2 coincided with an increased rate of tumour-cell apoptosis. These findings strongly support an antisense mechanism of action to increase the cytotoxic action of chemotherapy, although other therapeutically beneficial modes of action cannot be excluded.

Augmerosen includes CpG motifs, which have been purported to have anticancer effects in some animal models through immune stimulation, possibly of natural killer cells or a Th1 response.[23,24] To assess whether immune stimulation occurred during infusion, we tested peripheral blood lymphocytes at baseline and after 2 days of infusion in three patients treated with 6·5 mg/kg daily. No patient showed stimulation of natural killer cells (data not shown). Similarly, assessment of immune response after treatment of patients with non-Hodgkin lymphoma revealed no consistent change.[8] Notably, the major response in bulky metastatic melanoma, documented in our patient 12 (figure 3), was not associated with fever, flushing, or any cytokine-like symptoms that might be attributable to immune stimulation. Cytokine-like symptoms have been reported in phase I studies with other phosphorothioate antisense oligonucleotides; thus, these side-effects may be linked to the phosphorothioate backbone. Taken together, the results of clinical trials do not suggest that immune stimulation is a predominant mechanism for the antitumour effect of augmerosen. The data support an antisense mechanism for the activity of augmerosen and other phosphorothioate oligonucleotides.[8,9,14]

Most of our patients had progressive metastatic disease on entry, after failure of dacarbazine-containing regimens or after other standard treatments for metastatic melanoma. Even so, antitumour responses were noted in six of the 14 patients and stabilisation of disease was observed in two others. The estimated median survival of all 14 patients now exceeds 12 months. Despite the small number of patients, these results compare favourably with negligible response rates and median survival times of 4–5 months in patients with advanced melanoma after treatment failure of first-line systemic therapy.[1,2,25,26] Further understanding of the clinical usefulness of this combination regimen will be provided by results of an international randomised phase III trial that is comparing 5-day infusions of augmerosen (7 mg/kg daily) plus dacarbazine with single-agent dacarbazine.

Contributors

B Jansen was responsible for design, submission to the ethics committee, care of patients, administration of the study, and preparation of the report. V Wacheck contributed to protocol preparation and ethics committee submission, care of patients, training of research nurses, western blotting, study documentation, and preparation of the report. E Heere-Rees contributed to care of patients, study documentation, and the study protocol, and was responsible for detailed photodocumentation of skin lesions. H Schlagbauer-Wadl was responsible for western blotting and densitometric analysis of BCL2 expression and apoptosis assays, and contributed to the study protocol and preparation of study medication. C Hoeller contributed to care of patients (follow-up visits), study documentation, the study protocol, and histopathology. T Lucas contributed to FACS analysis, the study protocol, study documentation, training and supervision of laboratory technicians, sample preparation, and evaluation of pharmacokinetic analyses. M Hoermann undertook analyses of tumour lesions and contributed to study documentation and preparation of the study protocol. U Hollenstein contributed to care of patients (screening visits, chemotherapy rounds) and study documentation. K Wolff contributed to the study protocol and the report, care of patients, and histopathology. H Pehamberger contributed to the study protocol and the report, and was responsible for study amendments, care of patients, and histopathology.

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